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Isomerisation of phospho-BRCA1-BARD1 promotes Replication Fork Protection

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Summary

Genomic integrity is constantly threatened by problems encountered by the replication fork. BRCA1, BRCA2 and a subset of Fanconi Anaemia proteins protect stalled replication forks from nuclease degradation through pathways involving RAD51. The contribution and regulation of BRCA1 in replication fork protection, and whether this relates to, or differs from, BRCA1's role in homologous recombination (HR) is not clear. Here we show that the canonical BRCA1-PALB2 interaction is not required for fork protection but instead BRCA1-BARD1 is regulated through a conformational change mediated by the phosphorylation-directed prolyl isomerase, PIN1. PIN1 activity enhances BRCA1-BARD1 interaction with RAD51 and consequently RAD51's presence at stalled replication structures. We identify patient missense variants in the regulated BRCA1-BARD1 regions which show poor nascent strand protection but remain proficient for HR, defining novel domains required for fork protection associated with cancer development. Together these findings reveal a previously unrecognised pathway that governs BRCA1-mediated replication fork protection.

Main Text

Fork progression can be slowed by conflicts with transcription, deoxyribonucleotide (dNTP) shortage or by difficult to replicate sequences, frequently causing fork stalling¹. In order to prevent stalled forks collapsing into DNA double strand breaks (DSBs), a number of responses are elicited including fork remodelling and subsequent nascent strand protection. Agents that cause replicative stress or compromise DNA Polymerase- α function result in a proportion of forks reversing (reviewed in^{2,3}). The regressed arm of nascent DNA in reversed forks resembles a single-ended DNA DSB which is protected from excessive resection by RAD51. Several factors contribute to RAD51-mediated fork protection including BRCA1/2, FANCA/D2, RAD51 paralogs, BOD1L, SETD1A, WRNIP and Abro1².

One emerging theme is that stabilisation of the RAD51-nucleofilament is critical for protection of stalled forks⁴⁻⁶. RAD51 mutants that show increased dissociation are compromised in their ability to prevent fork degradation⁶⁻⁸ while RAD51-ssDNA stabilising factors, such as BOD1L, promote fork protection⁹. Other factors such as RADX, compete with, or dissociate, RAD51 so that their depletion rescues fork protection of BRCA-deficient cells^{10,11}. Failure to protect stalled forks is associated with genome instability. Restoration of replication fork protection in BRCA-deficient cells is linked to chemotherapy resistance in some cell types and contexts¹²⁻¹⁴. Thus further mechanistic understanding is needed to inform cancer patient care.

BRCA1 is found at ongoing and stalled replication forks^{15,16} and, like BRCA2, contributes to RAD51-mediated protection of stalled forks from MRE11-dependent degradation¹⁷. However, how BRCA1 contributes to fork protection is unclear. Here we show BRCA1 promotes the protection of nascent DNA at stalled replication forks independently of the canonical BRCA1-PALB2 interaction. We find that an enhanced, direct interaction between BRCA1-BARD1 and RAD51 is associated with nascent strand protection and this is dependent on the activity of the phosphorylation-directed prolyl isomerase, PIN1. We identify BRCA1 and BARD1 regions required for fork protection and find patient variants associated with familial and sporadic cancer which inhibit fork protection.

pS114-BRCA1 promotes fork protection

During homologous recombination (HR) repair of DNA double-strand breaks (dsDNA breaks), BRCA1-BARD1 regulates RAD51 localisation and loading through PALB2-BRCA2¹⁸⁻²⁰ but it is unclear if BRCA1 acts through this pathway in replication fork protection. We examined nascent DNA sequentially labelled with CldU-IdU at hydroxyurea (HU) stalled forks (5 mM HU, 3 hours)^{5 17} in PALB2 or BRCA1-depleted cells and both conditions showed decreased IdU/CldU ratios consistent with defective fork protection (Extended Data Fig 1a-c). Mutations in BRCA1 (M1411T) or PALB2 (Δ NT) coiled-coil motifs prevent direct interaction, fail to support HR-repair and show increased sensitivity to the inter-strand cross-linker Cisplatin^{19,20} (Extended Data Fig 1d-i). While both Flag-EGFP-M1141T-BRCA1 and Δ NT-PALB2 complemented cells showed increased fork stalling and defects in replication fork restart following HU exposure, these mutants were proficient in stalled replication fork protection (Extended data Fig 1j-o). Thus fork protection is independent of the canonical BRCA1-PALB2 interaction.

BRCA1 and BARD1 interact directly with RAD51^{21,22} and mutation of BARD1 residues F133, D135 and A136 (F133A-D135A-A136E, termed AAE) disrupts the BARD1-RAD51 interaction causing reduced resistance to mitomycin C²¹, Olaparib and HU (Extended Data Fig 2a-b). Complementation with RFP-Flag-AAE-BARD1 did not support protection of nascent strands unlike cells complemented with RFP-Flag-WT-BARD1 or the ubiquitin-ligase deficient mutant RFP-Flag-R99E-BARD1²³ (Extended Data Fig 2c-f). Thus protection of stalled replication forks requires the RAD51 binding site region in BARD1 but not heterodimer ligase activity.

BRCA1 and BARD1 are phosphorylated at residues potentially structurally close to the BARD1-RAD51 interaction site: S148-BARD1²⁴ and S114-BRCA1²⁵. Substitution of these sites to alanines (Flag-EGFP-S114A-BRCA1 or RFP-Flag-S148A-BARD1) reduced IdU/CldU ratios and CldU tract lengths following HU-treatment: both assays giving results consistent with defective fork protection (Fig 1a-b,

Extended Data Fig 3a-c). In contrast, mutation to the phospho-mimic aspartate (D) supported fork protection in cells complemented with Flag-EGFP-S114D-BRCA1 but not RFP-Flag-S148D-BARD1 (Fig 1a-b, Extended Data Fig 3d-e), supporting a role for BRCA1 phosphorylation in fork protection. Immunoblotting of RFP-Flag-S148D-BARD1 revealed the presence of a smaller BARD1 band (Extended Data Fig 3e), suggesting cleavage of this mutant.

Flag-EGFP-S114A-BRCA1 and Flag-EGFP-WT-BRCA1 showed similar levels of recruitment to active replication sites, comparable interactions with BARD1, and restoration of ongoing forks and fork restart after 3 hour exposure to 5 mM HU (Extended Data Fig 3f-i), suggesting that the S114-site is not significant to these aspects of replication stress. Mirin inhibition of MRE11 3'-5' nuclease activity in BRCA1/2 deficient cells restores fork protection¹⁷, and likewise Mirin-treatment restored long CldU tract lengths in Flag-EGFP-S114A-BRCA1 complemented cells (Extended Data Fig 3j-k), implicating the S114-site in the protection of nascent DNA from nuclease activity.

We generated an antibody against phosphorylated-S114 BRCA1 peptide (pS114) which detected immunoprecipitated Flag-EGFP-WT-BRCA1 but not Flag-EGFP-S114A-BRCA1 (Fig 1c). Intriguingly, BRCA1 phospho-S114 lies within an S-P motif which is a minimal consensus sequence for the phospho-peptidyl-prolyl isomerase (PPIase) PIN1, and BRCA1-BARD1 have previously been enriched from lysates using recombinant PIN1²⁶. We confirmed this interaction by immunoprecipitation of BARD1-BRCA1-PIN1 (Fig 1d). A GST-fusion of the PIN1 WW phospho-binding domain (GST-WW), but not a GST-W34A-WW mutant deficient in phospho-binding²⁷, could purify Flag-EGFP-WT-BRCA1 and Flag-EGFP-S114D-BRCA1 whereas neither GST-WW nor GST-W43A-WW purified Flag-EGFP-S114A-BRCA1 (Fig 1e and Extended Data Fig 4a-c). Furthermore, the interaction between exogenous or endogenous BRCA1 with recombinant GST-WW was increased following HU-treatment and lost following treatment of the cell lysate with phosphatase (Fig 1f, Extended Data Fig 4d-e). While RFP-Flag-WT-BARD1 was also purified by GST-WW, this interaction was not lost on mutation of the RAD51 proximal phosphorylation site at S148 (RFP-Flag-S148A-BARD1) (Extended Data Fig 4f). Together these data suggest that BRCA1 phosphorylation at S114 is upregulated in response to HU and promotes the ability of the GST-PIN1-WW domain to purify BRCA1.

To identify kinase(s) responsible for phosphorylating S114-BRCA1, we performed a targeted kinase inhibitor screen to members of the proline-directed kinase superfamily. Treatment with the CDK1/2 inhibitor, Roscovitine, but not inhibitors to JNK1/2 (SP600125), DYRKs (INDY), MEK1/2 (U0126) or GSK (Inhibitor IX), reduced the ability of GST-WW to enrich BRCA1 from cell lysates (Extended Data Fig 4g-h). Indeed, the BRCA1 S114 site lies within a loose CDK1/2 consensus site (S-P-x-x-K). Treatment of cells with the CDK1 inhibitor (RO-3306) or with CDK1 siRNA reduced the ability of the pS114 antibody or the GST-WW domain to purify BRCA1 from cell lysates (Fig 1g, Extended Data Fig 4i). Similarly incubation of recombinant CDK1/Cyclin A2 or CDK2/Cyclin A, but not CDK9/Cyclin K²⁸, with recombinant His-BRCA1₁₋₃₀₀-BARD1₂₆₋₁₄₂ resulted in phosphorylation of WT-BRCA1 at S114 (Extended Data Fig 4j-l). These data suggest that CDK1/2-mediated phosphorylation are likely to be the dominant kinases responsible for S114-BRCA1 modification.

Given the increase in pS114-BRCA1 following HU-treatment in cells, we next examined whether pS114-BRCA1 localizes to sites of stalled replication forks. Using the proximity ligation assay (PLA) we assessed potential proximity between EdU incorporated at ongoing forks, or at forks stalled by HU-treatment, with the phospho-specific pS114-BRCA1 antibody. We observed a BRCA1-dependent PLA

signal in HU-treated cells which was partially reduced in cells treated with a phosphatase (CIP) or the CDK1/2 inhibitor, Roscovitine (Fig 1h-i). Together these data support the view that pS114-BRCA1 is localised to stalled replication forks.

PIN1 regulates BRCA1-BARD1

PIN1 is enriched in isolated proteins on nascent DNA (iPOND) following treatment with HU¹⁰. We tested whether fork protection requires PIN1 and found that either PIN1 inhibition with Juglone, or PIN1-depletion, led to shortened CldU tracts and reduced IdU/CldU ratios following HU-treatment consistent with a fork protection defect (Fig 2a-b, Extended Data Fig 5a). Co-depletion of PIN1 with BRCA1 or BARD1 did not reduce nascent strand lengths further (Fig 2a-b, Extended Data Fig 5b-c). We saw no gross impact on BRCA1 or BARD1 protein levels following PIN1 depletion (Fig 2b, Extended Data Fig 5c-e).

In folded proteins, peptide bonds preceding residues other than proline (non-prolyl bonds) overwhelmingly favour the *trans* form, and *cis* bonds are rare in folded proteins²⁹. Due to the physical constraints of proline's unique 5-membered ring, peptide bonds preceding proline (prolyl bonds) may adopt the *cis* conformation³⁰. PIN1 is the only phospho-targeted PPlase that specifically recognises phospho-S/T-P motifs³¹ before catalysing a *cis-trans* conformational change of the peptidyl-prolyl bond³²⁻³⁵. To examine requirements for the *trans*-isomer one can substitute the target proline with another amino acid to favour the probability of the *trans* form^{36,37}. We mutated Flag-EGFP-BRCA1 P115 to an alanine or a cysteine and found both mutants maintained fork protection (Fig 2d-e, Extended Data Fig 5f-g), suggesting proline mutation or *trans*-isomerisation at this position is not deleterious. Importantly, inclusion of P115A or P115C with the S114A mutation restored IdU/CldU ratios in BRCA1-depleted cells to control levels (Fig 2d-e, Extended Data Fig 5f-g), showing that P115A or P115C can overcome the requirement for serine at 114. In cells co-depleted for BRCA1 and PIN1 we similarly found expression of Flag-EGFP-P115A-BRCA1 or Flag-EGFP-S114A-P115A-BRCA1, resulted in IdU/CldU ratios close to 1, compared to cells with Flag-EGFP-WT-BRCA1 expression (ratios of 0.5) (Fig 2f-g). Thus the requirement for PIN1 in fork protection can be largely overcome by expression of BRCA1 mutant at position P115.

BRCA1 isomerisation aids RAD51 binding

Both BRCA1 and BARD1 have RING-proximal regions significant to fork protection: BRCA1 contains the S114-P115 regulatory region and BARD1 contains a required RAD51 binding region. Given the potential physical proximity of these regions we next asked whether PIN1 influences BRCA1-BARD1-RAD51 interaction. Using recombinant proteins we first incubated WT His-BRCA1₁₋₅₀₀-BARD1₂₇₋₃₂₇ and phospho-mimic S114D His-BRCA1₁₋₅₀₀-BARD1₂₇₋₃₂₇ heterodimers with full length GST-PIN1 before incubating with purified, recombinant RAD51. Then we used His-pull down to examine the BRCA1-BARD1-RAD51 interaction. Only the heterodimer that included the BRCA1 phospho-mimic, S114D and which had been incubated with GST-PIN1 was able to bind RAD51 (Extended Data Fig 6a-b). To test this further we next phosphorylated His-BRCA1₁₋₃₀₀-BARD1₂₇₋₁₄₂ with CDK1/Cyclin A2 (Extended Data Fig 4j-l) and incubated this with either full length GST-WT-PIN1 or GST-C113S-PIN1 (Fig 3a and

Extended Data Fig 6b). C113S inhibits PIN1-mediated isomerisation³⁸, but does not prevent the interaction of PIN1 with its target proteins³⁹. Only phosphorylated His-BRCA1₁₋₃₀₀-BARD1₂₆₋₁₄₂ which had been incubated with active GST-WT-PIN1 was able to bind RAD51 (Fig 3a). These data confirm the requirement for phosphorylation and demonstrate the need for PIN1-activity in enhancing RAD51 binding to the BRCA1-BARD1 heterodimer. Finally, we generated recombinant purified WT and P115A His-BRCA1₁₋₅₀₀-BARD1₂₆₋₃₂₇ (Extended Data Fig 6c) and incubated these with RAD51 in the absence of PIN1. We found a greater interaction with RAD51 when the BRCA1-BARD1 heterodimer bearing P115A-BRCA1 was used (Fig 3b). These data are consistent with the finding that PIN1 activity is needed to increase RAD51 binding and also suggest that PIN1 presence is not directly needed to contribute to heterodimer-RAD51 interaction.

Since BRCA1 also contains a RAD51 binding region (aa708-1064), the impact of regulation around BRCA1 residue 115 may differ in the context of full-length proteins. Using full length BRCA1-BARD1 purified from insect cells we found that both WT and P115A-BRCA1-BARD1 interacted with RAD51 but the interaction was enhanced with heterodimers containing P115A-BRCA1 (Extended Data Fig 6d-e). Moreover immunoprecipitated P115A-BRCA1-BARD1 from mammalian cells showed a similar enrichment for RAD51 (~1.5-fold) (Extended Data Fig 6f-g). We exposed length recombinant WT-BRCA1-BARD1 to limited proteolysis and found that the rate of BRCA1 digestion was virtually unchanged by inclusion of P115A mutation. However, remarkably, the BARD1 digestion rate was increased by inclusion of P115A-BRCA1 (Fig 3c-d, Extended Data Fig 6h), indicating improved accessibility to BARD1 in the presence of P115A-BRCA1.

We addressed whether mutations of the S114-P115-BRCA1 site influence RAD51 presence at nascent DNA following HU-treatment^{4,5} using the proximity linked ligation assay (PLA)⁴⁰. Flag-EGFP-S114A-BRCA1 complemented cells were unable to restore WT-levels of RAD51-EdU PLA-foci in contrast to cells complemented with Flag-EGFP-S114A-P115A-BRCA1 (Fig 3e, Extended Data Fig 6i). These data suggest that the P115A mutant in BRCA1 overcomes the requirement for a functional phosphorylation site, or serine, at S114 to promote RAD51 accumulation at nascent DNA. Likewise, in cells co-depleted for BRCA1 and PIN1 the RAD51-EdU PLA foci levels were restored by complementation of Flag-EGFP-S114A-P115A-BRCA1 but not Flag-EGFP-S114A-BRCA1 (Fig 3f). Thus in cells PIN1's contribution to RAD51 recruitment at stalled forks can be overcome by P115A-BRCA1 expression.

Isomerisation promotes genome stability

Brca1 deficient murine B-cells manipulated to enable fork protection, while still retaining HR-deficiency, exhibit reduced chromosome aberrations following PARPi/Cisplatin treatment and show resistance to HU¹². This suggests loss of fork protection capability alone could be associated with altered responses to therapeutic agents. Cells complemented with Flag-EGFP-S114A-BRCA1 were resistant to PARP inhibitors (Olaparib, Veliparib and 4AN) and formed RAD51 foci after Olaparib treatment, but were sensitive to 16-hour treatment with replication stress-inducing agents (HU and Aphidicolin) (Fig 4a-b, Extended Data Fig 7a-g). Since prolonged HU treatment causes fork collapse and double strand breaks⁴¹, we also assessed colony survival in response to conditions that promote fork stalling but not collapse (3 hours, 5 mM HU)^{41,42}. Intriguingly, even in asynchronous cells treated with short-term HU, Flag-EGFP-S114A-BRCA1 complemented cells showed reduced colony formation

(Fig 4b-c). Moreover inclusion of the P115A mutation on the S114A background was sufficient to rescue the S114A defect in survival seen in response to both 3 hour and 16-hour treatment with HU (Fig 4a-c).

These data led us to investigate chromosome stability. Depletion of BRCA1 increased both the average number of breaks per metaphase and the percentage of metaphases with radial chromosomes (Fig 4d-f). Strikingly complementation with Flag-EGFP-S114A-BRCA1 restored the percentage of radial chromosomes, but not chromosome breaks, to control and Flag-EGFP-WT-BRCA1 levels, while complementation with Flag-EGFP-S114A-P115A-BRCA1 both phenotypes to control levels (Fig 4d-F). These data build a view in which the S114-P115 region of BRCA1 supports nascent DNA protection, the prevention of chromosome breakages, and HU resistance.

Constitutive P115A-BRCA1 is genotoxic

We next questioned the necessity for such a complex mechanism of BRCA1-BARD1 regulation and whether the constitutive presence of a heterodimer primed for increased RAD51 interaction and fork protection is deleterious. We constitutively expressed Flag-EGFP-WT-BRCA1 Vs Flag-EGFP-P115A-BRCA1 in cells over several days. Cells were taken at 4 day intervals and assessed for BRCA1 expression, γ H2AX foci as a marker of DNA damage signalling, and chromosome integrity in metaphase spreads (Fig 5a-d, Extended Data Fig 8a). Flag-EGFP-P115A-BRCA1 cells showed a significant increase in γ H2AX staining and the number of breaks per metaphase compared to matched Flag-EGFP-WT-BRCA1 cells (Fig 5a-d and Extended Data Fig 8a). Thus long-term exposure to P115A-BRCA1 promotes an increased rate of DNA damage accumulation.

Fork protection in patient variants

Somatic and germ line genetic variants associated with cancer have been found within or close to the BRCA1 S114-P115 phosphorylation-isomerisation site and the BARD1-RAD51 binding region (<https://research.nhgri.nih.gov/bic/>^{43,44 45}). To assess whether these affect replication fork protection, we generated point mutations corresponding to missense variants with a high Grantham difference and low variance (Fig 6a-b). *BRCA1* patient variants S114P, R133C, Y179C, S184C, S256Y, but not Y101N, and *BARD1* variants K144N and F147C, but not D135Y, showed reduced IdU/CldU ratios compared to controls consistent with a fork protection defect (Fig 6c-f). Moreover all mutants that showed a fork protection defect, also showed increased sensitivity to HU, while those that were proficient in fork protection did not (Fig 6g-i).

The S114A-BRCA1 mutation supports several aspects of BRCA1 function: it prevents quadra-radial formation thought to represent toxic-NHEJ of cells deficient in HR-repair⁴⁶, allows RAD51 foci, and supports PARPi resistance and replication restart (Fig 4d-f, Extended Data Fig 3i and 7b-g). These features suggest no significant role for the mutation in reducing HR repair proficiency. To address whether patient variants close to the S114-P115-BRCA1 and RAD51-BARD1 interaction regions show features of a recombination defect we examined complemented cells for RAD51 foci in S-phase cells after irradiation, cisplatin sensitivity and sensitivity to both short term (2 hour) and continuous exposure to Olaparib. Where possible cells expressing patient variants were also assessed for repair

of an integrated HR-substrate following enzymatic generation of a DNA DSB (Extended Data Figs 9-10 and summarised in Extended Data Table 1). Of those BRCA1 variants that showed poor fork protection and HU sensitivity S114P-BRCA1 and R133C-BRCA1 were not sensitive to continuous exposure to Cisplatin nor to short or continuous exposure to Olaparib and showed near WT-levels of RAD51 foci after Ionising Radiation (IR) (Extended Data Fig 9a-f). Of the BARD1 variants that showed poor fork protection and HU sensitivity K144N-BARD1 and F147C-BARD1 exhibited resistance to either length of Olaparib exposure, had near WT levels of RAD51 foci and showed resistance to all but the highest concentrations of Cisplatin. In addition, all BARD1 patient variants restored WT levels of HR in U2OS DR-GFP reporter cells depleted for BARD1 (Extended Data Fig 10a-f).

Discussion

Our data reveals a direct function for BRCA1 in fork protection that is separate from the canonical PALB2-BRCA2 recruitment. Instead we propose a new model of BRCA1-BARD1-RAD51 regulation and identify post-translational modifications of BRCA1 upregulated by HU-treatment required for fork protection. Our findings support a model in which CDK1/2-PIN1 regulated conformational change results in an improved interface between BRCA1-BARD1 and RAD51 (Extended Data Fig 11). While PIN1 activity is capable of disrupting dimers and aggregates and driving interactions specific for *cis* or *trans* conformations^{47,48}, this mechanism of isomerisation on one partner of a heterodimer promoting improved protein-protein interactions mediated by the other partner, appears unique. The cancer-associated patient mutations we identify in BRCA1-BARD1, extend the known regions of both proteins involved in fork protection. Our finding of Olaparib and cisplatin resistance of cells that express mutants which are specifically deleterious for fork protection are consistent with the resistances of murine cells bearing BARD1-BRCT mutations, which are deficient in nascent strand protection due to poor recruitment of the heterodimer to stalled forks⁴⁹. While our findings build on the view that greater RAD51 interactions promote fork protection, further investigation is required to explain how the increased RAD51 interaction with BRCA1-BARD1 specifically contributes to the function of RAD51 in fork protection. Our current ability to interrogate fork protection remains crude and measures observations seen after prolonged fork stalling which are unlikely to be physiologically directly relevant. Therefore, we require a greater understanding of fork protection mechanisms in order to accurately assess the relationship with therapeutic resistance/sensitivities.

Mice bearing *Bard1*-BRCT alleles that confer a fork protection defect are not tumour prone⁴⁹, and restoration of fork protection in a *Brca2* knock-out cancer model accelerates, rather than slows, tumour formation⁵⁰. Moreover HR, and not stalled fork protection, is associated with promoting human mammary cell viability¹⁴. Thus the balance of current evidence does not favour fork protection as a tumour suppression mechanism. Nevertheless here we report seven patient-derived variants in BRCA1-BARD1 that impair fork protection, four of which appear to have little impact on features of HR repair. These data provide a framework to address whether and how BRCA1-mediated fork protection relates to cancer development.

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Figure 1. pS114-BRCA1 is required for fork protection

a-b, IdU/CldU ratios from U2OS cells depleted for BRCA1, complemented with Flag-EGFP-BRCA1 variants and treated with HU (5 mM, 3 hours). n=205 fibres from 2 biological repeats, bars=median±95% Confidence Interval (CI). Representative western blot (n=3, **b**).

c, Detection of pS114-BRCA1 (rabbit polyclonal) in Flag-IPs from HEK293 cells expressing Flag-EGFP-WT-BRCA1 or Flag-EGFP-S114A-BRCA1 (representative blot n=3).

d, Representative Flag-IP from U2OS cells expressing RFP-Flag-BARD1 (n=3).

e, Representative image of GST-PIN1-WW pull down assay from U2OS cells expressing Flag-EGFP-WT-BRCA1 or Flag-EGFP-S114A-BRCA1. GST-W34A-WW-domain was used as a negative control. (n=4, data=mean±SEM)

f, Quantification of enrichment in GST-WW-domain pull downs from HEK293 cell lysates expressing Flag-EGFP-WT-BRCA1 treated ±HU (3 mM, 6 hours). (n=4, data=mean±SEM).

g, pS114-BRCA1 (rabbit polyclonal) was measured following Flag-IP of Flag-EGFP-BRCA1 from HEK293 cells treated with 5 µM CDK1/2 inhibitor RO-3306 (representative blot n=3).

h-i, Representative images (**h**) and quantification (**i**) of Proximity Ligation Assay (PLA) between pS114-BRCA1 (rabbit polyclonal) and EdU-Biotin in U2OS cells at ongoing (-HU) or stalled forks (+HU) pulse-labelled with EdU. Scale bars=10µm (**i**) Includes data from cells depleted for BRCA1 or treated with Calf Intestinal Phosphatase (CIP) or Roscovitine (25 µM). n=150 cells from 3 biological repeats. Bars=median±95% CI.

All *P*-values derived from two-sided unpaired *t*-test. For gel source data in this figure and throughout see Supplementary Figure 1.

Figure 2. PIN1 regulates BRCA1-BARD1 in fork protection

a-b, IdU/CldU ratios from U2OS cells depleted for BRCA1 and/or PIN1 and treated with 5 mM HU, 3 hours (n=300 fibres from 3 biological repeats, bars=median±95% CI). Representative western blot (n=3, **b**).

c, Schematic to illustrate *cis-trans* isomerisation around the BRCA1 phospho-S114 site.

d-e, IdU/CldU ratios from U2OS cells depleted for BRCA1, complemented with Flag-EGFP-BRCA1 variants and treated with 5 mM HU, 3 hours (n=320 fibres from 3 biological repeats, bars=median±95% CI). Representative western blot (n=3, **e**).

f-g, As for **d**, but in cells co-depleted for BRCA1 and PIN1 (n=195 fibres from 2 biological repeats, bars=median±95% CI). Representative western blot (n=2, **g**).

All *P*-values derived from two-sided unpaired *t*-test. n.s =not significant.

Figure 3. BRCA1 isomerisation enhances RAD51 binding

a, WT and CDK1-phosphorylated-His-BRCA1₁₋₃₀₀ and His-BARD1₂₆₋₁₄₂ incubated with full length GST-PIN1 to induce isomerisation or catalytically dead GST-PIN1-C113S as a control. Phospho-His-BRCA1-BARD1 complexes were incubated with recombinant active RAD51 and RAD51-binding was assessed by His-purification. Representative image n=3.

b, Recombinant WT or P115A His-BRCA1₁₋₅₀₀ and BARD1₂₇₋₃₂₇ were incubated with recombinant active RAD51 followed by His-purification to assess formation of the BRCA1-BARD1-RAD51 complex. Representative image n=5.

c-d, Percentage of remaining intact BRCA1 (**c**) or BARD1 (**d**) protein plotted against incubation time from trypsin digests of recombinant full length WT-BRCA1-BARD1 and P115A-BRCA1-BARD1

(Representative gel shown in Extended Data Fig 6h). Data=mean±SEM, n=3. k_p = rate constant of proteolysis ± SD.

e-f, RAD51-EdU PLA foci were measured in U2OS cells depleted for BRCA1 (**e**) or BRCA1 and PIN1 (**f**), complemented with Flag-EGFP-BRCA1 variants and treated with 5 mM HU, 4 hours.

Data=mean±SEM, n=3.

All *P*-values derived from two-sided unpaired *t*-test. n.s =not significant.

Figure 4. Loss of BRCA1-isomerisation leads to genome instability

a-c, Colony survival following HU-treatment (16 hours, n-shown in brackets, **a**) or (5mM, 3 hours, n=3, **c**) was measured in HeLa cells depleted for BRCA1 and complemented with Flag-EGFP-BRCA1 variants. Data=mean±SEM. Representative western blot (n=3, **b**).

d-f, Metaphase spreads from U2OS cells depleted for BRCA1, complemented with Flag-EGFP-BRCA1 variants and treated with HU (5mM, 4 hours). Representative images (**d**). Red arrows indicate chromosome and chromatid breaks. Blue asterisks mark radials. Quantification of chromosome or chromatid breaks per metaphase (n=80 metaphases from 4 biological repeats. Data=mean±SEM, **e**) and percentage of metaphases showing 1 or more radial chromosomes. Data=mean±SEM, n=4 (**f**)

All *P*-values derived from two-sided unpaired *t*-test.

Figure 5. Constitutively expressed P115A-BRCA1 is genotoxic

a, Accumulation of γH2AX damage foci in EdU-positive U2OS cells constitutively expressing Flag-EGFP-WT-BRCA1 or Flag-EGFP-P115A-BRCA1 over time. n=90 cells from 3 biological repeats.

Bars=mean±SEM.

b-d, Metaphase spreads from U2OS cells constitutively expressing Flag-EGFP-WT-BRCA1 or Flag-EGFP-P115A-BRCA1 for 28-36 days. Representative images from day 36 (**b**). Chromosome breaks marked by red arrows. Quantification of metaphase breaks n=80 metaphases from 3 biological repeats. Bars=mean±SEM (**c**). Representative western blot (n=3, **d**).

All *P*-values derived from two-sided unpaired *t*-test. n.s =not significant.

Figure 6. Loss of fork protection in BRCA1-BARD1 patient variants

a-b, Schematics showing BRCA1 (**a**) and BARD1 (**b**) marking the BRCA1-S114-P115 phosphorylation-isomerisation site, the BARD1-RAD51 binding domain and proximal patient variants with a class-65 Grantham score.

c-f, IdU/CldU ratios were measured from U2OS cells depleted for BRCA1 (**c**) or BARD1 (**d**) and complemented with Flag-EGFP-BRCA1 (**c**) or RFP-Flag-BARD1 (**d**) variants and treated with HU (5 mM, 3 hours). n=300 fibres (BRCA1); n=275 fibres (BARD1) both from 3 biological repeats, bars=median±95% CI. Representative western blot for BRCA1 (n=3, **e**) and BARD1 (n=3, **f**) variants.

g-i, Colony survival following HU-treatment (16 hours) was measured in HeLa (**g**) or U2OS cells (**h-i**) depleted for BRCA1 (**g-h**) or BARD1 (**i**) and complemented with Flag-EGFP-BRCA1 (**g-h**) or RFP-Flag-BARD1 (**i**) variants. Data=mean±SEM. n-values given in brackets for each condition.

All *P*-values derived from two-sided unpaired *t*-test.

Methods (online only)

Site-Directed Mutagenesis. Specific primers were designed for mutagenesis (Supplementary Table 1) and mutagenesis performed by PCR using PfU (Promega). All mutagenesis was confirmed by Sanger Sequencing (Source Bioscience).

Tissue culture. Parental Flp-InTM HeLa, U2OS and HEK293 cells were obtained from Morris Lab cells stocks and grown in Dulbeccos Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. Cells were cultured in Corning T75 flasks and 10 cm² plates and kept at 5% CO₂ and 37 °C. Once cells reached 70-80% confluency they were passaged. Cells were tested for Mycoplasma by Hoescht staining. Cell lines have not been authenticated.

Inducible stable cell line generation. Stable cell lines were generated from Flp-InTM HeLa, U2OS and HEK293 cells cells co-transfected with the gene of interest cDNA in the pcDNA5/FRT/TO vector and the Flp recombinase cDNA in the pOG44 vector. Control transfections were carried out without the pOG44 recombinase. Two days after transfection, cells were selected with 100 µg/ml Hygromycin, cell culture medium was replaced every 2-3 days and cells were selected for approximately 2 weeks. After selection cells were expanded and tested for expression of Flag-EGFP-BRCA1, RFP-Flag-BARD1 or Flag-PALB2. Cells were treated with 2 µg/ml Doxycycline for 24, 48 and 72 hours and expression levels were checked by western blotting.

Plasmid and siRNA transfection. FuGENE 6 (*Roche*) was used as a reagent to transfect DNA plasmids into cells, the ratio used was 4:1 FuGENE (µl): DNA (µg), following the manufacturer's guidelines. siRNA transfections were carried out using the transfection reagent Dharmafect1 (*Dharmacon*) following the manufacturer's instructions. For a full list of siRNA sequences see Supplementary Table 2.

Colony survival assays. Flp-InTM U2OS or HeLa cells were plated in 24 well plates at 4 x 10⁴ cells/ml and treated according to the experiment performed. Cells were trypsinised in 100 µl of 1x Trypsin and resuspended in 900 µl of PBS. Cells were plated out at limiting dilutions and incubated for a further 10-14 days at 37 °C at 5 % CO₂. Once colonies had grown they were stained with 0.5 % Crystal violet in 50 % methanol and counted. For a full list of DNA damaging agents and inhibitors, see Supplementary Table 3.

GST-PIN1-WW Pull down assay. Cells were washed with 10 ml ice cold PBS before being lysed in 5 ml TG lysis buffer (40 mM Tris-HCL pH 8, 274 mM NaCl, 2 mM EGTA, 3 mM MgCl₂, 2 % Triton x100, 20 % Glycerol) with addition of cOmplete protease inhibitor cocktail (Roche) and PhosSTOP (Roche) tablets. The lysed cells were transferred into 1.5 ml Eppendorf tubes incubated on ice for 20 mins and sonicated twice at 20 % intensity for 10 seconds. Samples were spun at 13000 rpm at 4 °C for 10 mins and the supernatant kept. 50 µl of the supernatant was mixed with 20 µl 4x SDS Loading buffer and boiled at 95 °C for 5 mins. 800 µl of the cell supernatant was then incubated with equal concentrations of GST-WW PIN1 and GST-W34A PIN1 beads for 2 hours at 4°C. The GST-pull downs were washed three times in TG lysis buffer before adding 60 µl 4x SDS loading buffer directly to the beads. Samples were boiled at 95 °C for 5 mins and then 40 µl loaded onto an SDS PAGE gel and analyzed by western blotting.

Flag immunoprecipitation. Stable U2OS or 293 cells were plated in a 10 cm² plate and treated with

Doxycycline for 48 hours to express inducible Flag-EGFP-BRCA1 or RFP-Flag-BARD1 or 293 cells were transiently transfected with 4 µg pcDNA5/FRT/TO-Flag-EGFP-BRCA1 and/or pcDNA5/FRT/TO-RFP-Flag-BARD1 for 48-72 hours. Cells were washed with 10 ml ice cold PBS before being scraped in ice cold Nuclear Lysis Buffer (10 mM HEPES pH7.6, 200 mM NaCl, 1.5 mM MgCl₂, 10 % Glycerol, 0.2 mM EDTA, 1 % Triton) with addition of cOmplete protease inhibitor cocktail (Roche) and PhosSTOP (Roche) tablets, 1 U/ml DNase1 (ThermoFisher) and 20 µM MG132 for every 10 ml. The lysed cells were then transferred into 1.5 ml Eppendorf tubes incubated on ice for 30 mins. Samples were spun at 13000 rpm at 4°C for 10 mins and the supernatant kept, the pellet was discarded. 50 µl of the supernatant was mixed with 20 µl 4x SDS Loading buffer and boiled at 95°C for 5 mins. For every IP, 10 µl Flag-agarose beads were firstly washed out of storage buffer by doing 3x 1ml PBS washes and centrifuging at 3000 rpm between each wash. 90 µl of PBS was added for every 10 µl of agarose beads. Once the beads were resuspended in PBS, 100 µl were transferred into an Eppendorf with 500 µl of supernatant and 500 µl of PBS. The Eppendorfs were rotated for 2 hours at 4°C. Samples were centrifuged at 3000 rpm for 1 min and the beads left to settle. The supernatant was then removed before 3x 1 ml PBS-0.02% tween washes. The wash buffer was completely removed before adding 60 µl 2x SDS loading buffer. This was boiled at 95°C for 5 mins and then 30 µl loaded onto an SDS PAGE gel and analysed by western blotting.

Western blotting. For a full list of antibodies see Supplementary Table 4. Samples were run on acrylamide SDS PAGE protein gels and transferred to PVDF Immobilon-P membrane. Following transfer membranes were blocked in 5 % marvel milk in PBS with 0.1 % Tween (PBStw) or in 5 % BSA with PBStw, for a minimum of 1 hour before incubation with primary antibody at 4 °C for 16 hours. Blots were washed 3x 10 mins in PBStw and then transferred into secondary HRP antibodies in 5 % marvel milk for a minimum of 1 hour. Blots were again washed 3x 10 mins in PBStw before probing with 1:1 EZ-ECL mix (Biological Industries). Blots were exposed to X-Ray film (Wolff Labs) and developed using the Xograph Compact X4 developer. Densitometry calculations were performed using Image J. Source data for uncropped gel images for western blots are available in Supplementary Figure 1.

p-S114 Antibody Generation. Custom mouse monoclonal (3C10G8) and rabbit polyclonal antibodies were raised against BRCA1 phospho-S114 by Genscript using the following peptide: CFAKKENNpSPEHLKD. These antibodies are available on request to the corresponding authors subject to completion of a standard MTA.

Fibre labelling and spreading. Cells were seeded in 6 cm² plates at a density of 20x 10⁴ cells/well and treated with thymidine analogues CldU and IdU. To monitor stability of nascent DNA, cells were incubated at 37 °C with CldU for 20 mins at a final concentration of 25 µM, followed by incubation with IdU (250 µM) for 20 mins and then with 5 mM HU for 3 hours. To monitor CldU fibre lengths, cells were incubated at 37 °C with CldU for 20 mins at a final concentration of 25 µM and then with 5 mM HU for 3 hours. To monitor replication fork restart, cells were incubated at 37 °C with CldU for 20 mins at a final concentration of 25 µM and then with 5 mM HU for 3 hours. The HU was then washed out with 3x PBS washes and cells were incubated for a further 40 mins in media containing 250 µM IdU at 37°C.

After incubation with thymidine analogues, cells were washed 2x with ice-cold PBS for 5 minutes with rotation then trypsinised, resuspended in 1ml of PBS and counted. The optimal concentration is

50×10^4 cells/ml and thus cells were adjusted to such concentration. 2 μ l of the cell sample was placed on Snowcoat microscope slides and allowed to slightly dry for 7 mins. Then 7 μ l of spreading buffer (200 mM Tris pH7.4, 50 mM EDTA, 0.5% SDS) was mixed with the sample and incubated for 2 mins to lyse the cells. In order to spread the sample down the slide, slides were gradually tilted and once the sample had reached the bottom of the slide, they were allowed to dry for 2 mins. Finally, slides were fixed in a 3:1 ratio of Methanol: Acetic acid for 10 mins before leaving slides to air dry for 5-10 mins. Dried slides were stored at 4°C till staining.

Fibre Immunostaining. After fibre spreading slides were washed twice for 5 minutes with 1 ml H₂O and rinsed with 2.5 M HCl before denaturing DNA with 2.5 M HCl for 1 hour 15 mins. Slides were then rinsed 2 x with PBS and washed for 5 minutes in blocking solution (PBS, 1 % BSA, 0.1 % Tween20). Slides were incubated for 1 hour in blocking solution. After blocking, each slide was incubated with 115 μ l of primary antibodies, Rat α BrdU (AbD Serotec/Abcam) to detect CldU used at a concentration of 1:2000 and Mouse α BrdU (Becton Dickinson) to detect IdU used at 1:750. Slides were covered with large coverslips and incubated with the antibodies for 1 hour. After incubation with the primary antibody, slides were rinsed 3x with PBS and then incubated for 1 min, 5 mins and 30 mins, with blocking solution. After rinsing and washing, slides were incubated with 115 μ l of secondary antibodies (α -Rat AlexaFluor 555 and α -Mouse AlexaFluor 488) in blocking solution, at a concentration of 1:500, covered with a large coverslip for 2 hours. Slides were rinsed 3x with PBS and incubated with blocking solution for 1 min, 5 mins and 30 mins. After again rinsing 2 x with PBS, immunomount mounting media was added to the slide and a large coverslip placed over and left to dry. Coverslips were then stored at -20°C for microscopy analysis. It is important to point out that during this process slides must be kept protected from light.

Following imaging using a Leica DM6000B fluorescence microscope, CldU and IdU fibre lengths were measured for ongoing forks containing both labels using Image J. The ratio of IdU/CldU was determined for each ongoing fork for a minimum of 100 fibres per experiment and each experiment was repeated 3 times.

Fork restart was measured as the percentage of fibres with incorporation of both labels (CldU & IdU). Stalled forks were fibres with only first label (CldU). Percentage was calculated accordingly to the total number of fibres counted.

Immunofluorescent staining. U2OS WT or S114A-BRCA1 cells were plated at a density of 5×10^4 cells/ml in 24 well plates on circular glass coverslips (13 mm). Cells were treated siBRCA1 for 48 hours and complemented with WT or S114A BRCA1 by addition of Doxycycline. Cells were then treated with 20 μ M Olaparib for 2 hours. Cells were pre-permeabilised by incubation with CSK buffer (100 mM sodium chloride, 300 mM sucrose, 3 magnesium chloride, 10 mM PIPES pH 6.8) on ice for 1 minute before fixation with 4 % PFA. Once fixed the cells were permeabilised for a further 5 mins using 0.5 % TritonX in PBS before incubation with blocking solution - 10% FCS in PBS for 30 mins. Cells were then incubated with rabbit polyclonal RAD51 (H92) antibody in 10% FCS PBS at room temperature overnight. The following day, cells were washed in PBS/FCS before incubating them with AlexaFluor-488 secondary antibody at a 1:2000 concentration, for 2 hours. Cells were then washed three times in PBS and the DNA stained using Hoescht at a 1:20000 concentration for 5 mins. Excess of Hoescht was washed with PBS and coverslips mounted onto Snowcoat slides using Immunomount mounting media.

EdU staining. Cells subjected to immunofluorescent staining were also subjected to EdU staining. Cells were incubated with 10 μ M final concentration of EdU for 2 hours prior to fixing and staining was carried out as detailed in the Click-it® EdU Imaging Kits (Life Technologies).

CldU Immunostaining. Cells were plated at a density of 5×10^4 cells/ml in 24 well plates on circular glass coverslips (13 mm). Cells were treated as described and incubated at 37 °C with CldU for 20 mins at a final concentration of 25 μ M. Cells were then fixed with 4 % PFA and permeabilised for 5 mins using 0.25 % TritonX in PBS. After permeabilisation, cells were washed twice with PBS and twice with blocking solution containing 10 % FCS in PBS. 2 M HCl was added for 30 mins at 37 °C and cells incubated with 10 % FCS in PBS for 1 hour at room temperature (RT). Cells were then incubated with anti-Flag (M2-Sigma)(1:1000) and Rat α BrdU (AbD Serotec) (1:500), in 10 % FCS PBS at RT, for 1 hour. After primary antibody incubation, cells were washed 3x in PBS and then incubated for 10 mins in stringency buffer (0.5 M NaCl, 36 mM Tris pH 7.5-8, 0.5 % Tween20), before incubating them with AlexaFluor antibodies at a 1:500 concentration, for 2 hours. Cells were then washed twice with PBS and once with stringency buffer, and DNA stained using Hoescht at a 1:20000 concentration for 5 mins. Excess of Hoescht was washed with PBS and coverslips mounted onto Snowcoat slides using Immunomount mounting media.

Proximity Ligation Assay. Flp-In™ U2OS cells were seeded at 4×10^4 cells/ ml onto poly-L-lysine coated coverslips and EdU pulsed for 10 minutes at 37°C for 10 minutes. 5 mM HU was then added into media for 4 hours at 37 °C. Likewise, cells were treated with Roscovitine (25 μ M) for 4 hours at 37 °C. Cells were pre-extracted for 5 minutes on ice with Pre-extraction buffer (20 mM NaCl, 3 mM MgCl₂, 300 mM Sucrose, 10 mM PIPES, 0.5 % Triton X-100) and fixed in 4 % PFA for 10 minutes before blocking in 10 % BSA for 16 hours. Blocking media was removed and click it reaction cocktail (PBS, 10 μ M Biotin Azide, 10 mM sodium ascorbate, 1 mM CuSO₄) was added for 1 hour at room temperature. Click it reaction was washed and cells blocked in 10 % BSA for 30 minutes. Cells were then incubated with primary antibodies, Biotin (Jackson Immunoresearch) and RAD51 (Calbiochem) or pS114-BRCA1 (rabbit polyclonal) in 5 % FCS in PBS for 1 hour at room temperature. After incubation with primary antibodies cells were incubated with the MINUS/PLUS PLA probes (Sigma Duolink PLA kit) for 1 hour at 37 °C in a warm foil covered box. Cells were then washed twice for 5 minutes with wash buffer A (Sigma Duolink PLA kit) and incubated with the Sigma Duolink Ligation kit (1X ligation buffer, ligase enzyme) for 30 minutes at 37 °C. Cells were washed twice for 5 minutes with wash buffer A and incubated for 100 minutes at 37 °C with the Sigma Duolink amplification kit (1X amplification buffer, polymerase enzyme). Finally they were washed twice for 10 minutes with wash buffer B (Sigma Duolink PLA kit) at room temperature and coverslips were mounted using the Duolink mounting media with DAPI (Sigma). For cells treated with Calf Intestinal Phosphatase (CIP) (NEB), fixed cells were re-permeabilised with 0.2 % TritonX100 in PBS for 5 minutes before incubation with 20 U of CIP for 20 minutes at 4 °C. Cells were then blocked and assayed as described above.

Microscopy. Immunofluorescent staining was imaged using the Leica DM6000B microscope using a HBO lamp with 100W mercury short arc UV bulb light source and four filter cubes, A4, L5, N3 and Y5, which produce excitations at wavelengths 360 488, 555 and 647 nm respectively.

Metaphase spreads. Flp-In™ U2OS cells were treated with 5 mM HU for 4 hours and then incubated with Colcemid (0.05 μ g/ml) 16 hours. Cells were trypsinized and centrifuged at 1200 rpm for 5

minutes. Supernatant was discarded and cells resuspended in PBS. 5 ml of ice-cold 0.56 % KCl solution was added and incubated at room temperature for 15 min before centrifuging at 1200 rpm for 5 min. Supernatant was discarded and cell pellet broken before fixation. Cells were then fixed in 5 ml of ice-cold methanol: glacial acetic acid (3:1). Fixation agents were removed and 10 µl of cell suspension was dropped onto an acetic acid humidified slide. Slides were allowed to dry for at least 24 hours and then stained with Giemsa solution (Sigma) diluted 1:20 for 20 min. Slide mounting was performed with Eukitt (Sigma).

Protein expression and purification

WT and P115A His-BRCA1₁₋₅₀₀-BARD1₂₇₋₃₂₇ for in vitro analysis The expression of His-BRCA1-WT + BARD1-WT and His-BRCA1-PA + BARD1-WT in Rosetta™(DE3) was induced by the addition of 1 mM Isopropyl-β-d-thiogalactopyranoside (IPTG), and the proteins were produced in LB medium containing 50 µg/ml of kanamycin, 100 µg/ml of ampicillin and 30 µg/ml of chloramphenicol at 37°C for 5 hours. For purification of the His-BRCA1-WT + BARD1-WT and His-BRCA1-PA + BARD1-WT products, the cells were harvested and resuspended in 20 mM HEPES potassium salt, pH 7.4, 50 mM Imidazole, 500 mM NaCl, 1.0 mM TCEP [tris(2-carboxyethyl)phosphine], cOmplete EDTA-free protease inhibitor cocktail tablet (Roche). Cells were lysed using an Emulsiflex-C3 homogenizer (Avestin) and broken by three passages through the chilled cell. The lysate was centrifuged at 75,000 xg using a JA 25.50 rotor (Beckman Coulter) and filtered through a 0.45-µm filter. The clarified lysate was applied onto a 5-ml HisTrap HP column (GE Healthcare). The column was washed extensively using the same buffer, and the protein was eluted using buffer containing 500 mM imidazole.

Fractions containing a band of the correct size were concentrated using a Vivaspin 20-ml concentrator (10,000 molecular weight cut-off [MWCO]) (GE Healthcare) and gel purified using an Akta Pure 25 (GE Healthcare LS) with a prepacked Hi-Load 10/300 Superdex 200 PG column.

WT and S114A BRCA1₁₋₃₀₀-HisBARD1₂₆₋₁₄₂ for CDK kinase assay. BRCA1 and BARD1 proteins were expressed from pET15b-His-BRCA1₁₋₃₀₀:His-BARD1₂₆₋₁₄₂ vector in BL21(DE3) bacteria (Bioline). Bacteria were grown at 37 °C until an optical density of 0.6 was reached. Protein expression was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Bioline), and the temperature was immediately decreased to 25 °C. Bacteria were grown for a further 24 h. Bacterial pellets were collected after centrifugation at 3,000g for 10 min at 4 °C and then lysed in ice-cold lysis buffer (50 mM sodium phosphate, pH 7, 300 mM sodium chloride, 5% glycerol and 10 mM β-mercaptoethanol). Lysates were sonicated for 1 min at 30 % intensity and then clarified by centrifugation at 14000g for 10 min at 4 °C. The supernatant was incubated with 0.25 ml His-select beads (Sigma) overnight at 4 °C with rotation. The following day, the beads were washed three times with ice-cold wash buffer (50 mM sodium phosphate, pH 7, 300 mM sodium chloride, 5 % glycerol, 10 mM β-mercaptoethanol and 50 mM imidazole) before elution on ice in 50 mM sodium phosphate, pH 7, 300 mM sodium chloride, 5 % glycerol, 10 mM β-mercaptoethanol and 300 mM imidazole. Purified proteins were dialyzed against (25 mM Tris-HCl, pH 7.5, 10 % glycerol, 2 mM dithiothreitol (DTT) and 150 mM potassium chloride), and purity was assessed by resolution on a 15 % SDS–PAGE gel.

GST-PIN1 and GST-PIN1-WW purification. BL21 Escherichia Coli were transformed with the pGEX protein expression vector containing either full length PIN1 or the WW domain of PIN1 (WT or W34A). Colonies were picked and grown up in 50 ml starter cultures containing Ampicillin at 37°C for 16 hours at 200 rpm. Starter cultures were transferred to 500 ml Luria Bertani (LB) containing Amp and grown for 2 hours at 37 °C at 200 rpm. Bacterial expression was induced using 0.5 mM IPTG and bacteria left to grow for 5 hours at 37°C at 200 rpm.

Bacteria were pelleted by centrifuging at 12000 rpm for 10 mins at 4°C. Then the bacterial pellet was lysed in 10 ml GST lysis buffer (20 mM Tris-HCL pH8, 130 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 1 % Tritonx-100, 10 % Glycerol, 1 mM DTT) with 1 protease inhibitor tablet (Roche). Bacteria were resuspended and left on ice for 20 mins and then sonicated 2 times at 20% intensity for 10 seconds. Lysed bacteria were spun 20 mins at 13000 rpm to pellet debris. Supernatant was transferred to a 50 ml falcon tube and made up to 35 ml with lysis buffer and 500 µl of pre-washed Glutathione Sepharose 4B beads (GE Life Sciences) and rotated at 4°C for 16 hours.

CDK Kinase assays. 50 ng of WT or S114A His-BRCA1₁₋₃₀₀:His-BARD1₂₆₋₁₄₂ were incubated with 50ng of recombinant CDK1-Cyclin A2 (Thermofisher PV6280), CDK2-Cyclin A (Thermofisher PV3267) or CDK9/Cyclin K (Thermofisher PV4335) in 25 mM MOPS pH7.2, 12.5 mM β-glycerolphosphosphate, 25 mM magnesium chloride, 5mM EGTA, 2 mM EDTA, 0.5 mM DTT and 50 ng/µl BSA. The kinase reaction was started by addition of 10 mM ATP and samples were incubated at 30 °C for 30 minutes. The reaction was stopped by addition of 4x SDS-PAGE loading buffer and incubated at 95 °C for 5 minutes. 5 µl of the total reaction was run on 14 % SDS PAGE gel before transfer to Immobilon-P membrane (MERCK-Millipore) and western blotting for phospho-S114 (3C10G8) or BRCA1 (MS110).

RAD51 *in vitro* binding assay (BRCA1-BARD1 N-terminal fragments) 0.5 µl of Human recombinant RAD51 (ab63808 - Abcam) was incubated with 40 µl of a 50% slurry of His-BRCA1-WT + BARD1-WT or His-BRCA1-PA + BARD1-WT immobilized in Ni²⁺-resin together with 500 µl of RAD51 binding buffer (25 mM Tris-HCl pH 7.5, 10% Glycerol, 0.5 mM EDTA, 0.05% Igepal CA-630, 1mM 2-mercaptoethanol, 150 mM KCl, 50mM Imidazole) for 30 minutes at 4°C in rotation. After incubation the resin was washed three times with the RAD51 binding buffer before eluting in 40 µl 4x SDS loading buffer at 95°C for 5 minutes. The SDS elute was then analyzed by Western blotting and Coomassie blue staining.

***In vitro* PIN1 incubations with BRCA1-BARD1 and RAD51** Using recombinant BRCA1 preps 50 µl of HisBRCA1₁₋₅₀₀-BARD1₂₇₋₃₂₇ WT or the phosphomimic S114D was incubated at 37 °C for 1 hour with 2 ul of full length GST-PIN1 or BSA in 10 mM ATP in 50 mM Tris pH7.4, 500mM NaCl, 1 mM DTT, 500 mM Imidazole, 5 % glycerol and 0.01% Igepal CA630. This was then incubated for a further 2 hours at 4 °C with 0.5 µl of Human recombinant RAD51 (ab63808 - Abcam) in 500 µl of RAD51 binding buffer (25 mM Tris-HCl pH 7.5, 10% Glycerol, 0.5 mM EDTA, 0.05% Igepal CA-630, 1mM 2-mercaptoethanol, 150 mM KCl, 50mM Imidazole) and 30 µl of His-Select beads (Sigma). Beads were then washed 3-times in RAD51 binding buffer before eluting in 60 µl 4x SDS loading buffer at 95°C for 5 minutes.

Alternatively, 0.3 µg of WT HisBRCA1₁₋₃₀₀-HisBARD1₂₆₋₁₄₂ was incubated with or without 100 ng of recombinant CDK1-Cyclin A2 (Thermofisher PV6280), in 150 µl of 25 mM MOPS pH7.2, 12.5 mM β-glycerolphosphosphate, 25 mM magnesium chloride, 5mM EGTA, 2 mM EDTA, 0.5 mM DTT and 50 ng/µl BSA with 20 mM ATP at 37 °C for 2 hours. Subsequently, 50 µl BSA, or WT HisBRCA1₁₋₃₀₀-HisBARD1₂₆₋₁₄₂ or CDK1-phosphorylated HisBRCA1₁₋₃₀₀-HisBARD1₂₆₋₁₄₂ was incubated with 2 ul of full

length GST-PIN1 or BSA in 10 mM ATP in 50 mM Tris pH7.4, 500mM NaCl, 1 mM DTT, 500 mM Imidazole, 5 % glycerol and 0.01% Igepal CA630 for 5 minutes at room temperature. This was then incubated for a further 2 hours at 4 °C with 0.5 µl of Human recombinant RAD51 (ab63808 - Abcam) in 500 µl of RAD51 binding buffer (25 mM Tris-HCl pH 7.5, 10% Glycerol, 0.5 mM EDTA, 0.05% Igepal CA-630, 1mM 2-mercaptoethanol, 150 mM KCl, 50mM Imidazole) and 30 µl of His-Select beads (Sigma). Beads were then washed 3-times in ice cold PBS before eluting in 60 µl 4x SDS loading buffer at 95°C for 5 minutes.

Full length BRCA1-BARD1 cloning and purification. *His-BRCA1-Strep* and *BARD1* genes were synthesised with codons optimised for insect cell expression. Mutagenesis was carried out by PCR using CloneAmp HiFi PCR premix (Takara) and the primers indicated in Supplementary Table 1. Mutagenesis was confirmed by Sanger sequencing (Eurofins). Genes were cloned into the MultiBac system for expression in insect cells⁵¹.

BRCA1-BARD1 complexes were expressed in Sf9 insect cells for 60 hours post-infection in Insect-XPRESS Protein-free Insect Cell Medium with L-Glutamine (Lonza). Cells were harvested and resuspended in lysis buffer (50 mM NaPO₄, pH 8.0, 150 mM KCl, 0.5% NP-40, 0.01% Tween-20, 10% glycerol, 0.5 mM TCEP), BaseMuncher (Expedeon, UK), 1 mM benzamidine, and protease inhibitors (AEBSF 1 mM, aprotinin 800 nM, bestatin 50 µM, E-64 15 µM, leupeptin 20 µM, pepstatin 10 µM). The lysate was clarified by centrifugation at 36,000 x g for 1 hour at 4°C, passed through a 0.45 µm syringe filter and loaded onto a 5 mL StrepTrap HP column (GE Healthcare, USA). The column was washed with lysis buffer followed buffer A (50 mM NaPO₄, pH 8.0, 150 mM KCl, 0.01% Tween-20, 10% glycerol, 0.5 mM TCEP) and then buffer B (50 mM NaPO₄, pH 8.0, 50 mM KCl, 0.01% Tween-20, 10% glycerol, 0.5 mM TCEP). The protein was eluted onto a 1 mL HiTrap SP HP column (GE Healthcare) using elution buffer (wash buffer B supplemented with 0.4 mg/ml d-Desthiobiotin (Sigma, USA)). The SP column was washed with buffer C (25 mM HEPES, pH 7.4, 0.01% Tween-20, 10% glycerol, 0.5 mM TCEP) supplemented with 50 mM KCl. The protein was then eluted using a 0.05 – 1 M KCl gradient in buffer C. Peak fractions were pooled and concentrated with an Amicon 100 kDa MWCO centrifugal filter unit (Sigma). The concentrated protein was loaded onto a Superose 6 Increase 10/300 column (GE Healthcare) and gel filtrated in buffer C supplemented with 150 mM KCl. Peak fractions were pooled, flash frozen in liquid nitrogen, and stored at -80°C.

RAD51 purification (for binding assays to full length BRCA1-BARD1). RAD51 was purified according to the protocol described by Subramanyam⁵². Briefly, RAD51 was expressed from Acella DE3 *E. coli* induced with IPTG for 4 hours at 37°C. The pellet was resuspended in lysis buffer (100 mM Tris-OAc, pH 7.5, 2 mM EDTA, 10% glycerol, 1 mM DTT, protease inhibitor cocktail, lysozyme 0.5 mg/mL, 0.1% Triton X-100). The resuspended cells were sonicated and the lysate clarified by centrifugation at 18,000 rpm at 4°C for 1 hr 20 min. The supernatant was dialysed in spermidine acetate buffer (20 mM Tris-OAc pH 7.5, 7 mM spermidine, 5% glycerol, 0.1 mM DTT). RAD51 was selectively resuspended in buffers containing an increasing concentration of NaCl. The fractions were then purified with a HiTrap Q HP column (GE Healthcare).

RAD51 *in vitro* binding assays (full length BRCA1-BARD1)

BRCA1-BARD1 (0.5 µM) was pre-incubated with RAD51 (5 µM, gifted by Yueru Sun) for 30 min at 4°C. 80 µL of a 50% slurry of Streptactin Sepharose High Performance Resin (GE Healthcare) in binding buffer (25 mM Tris-HCl, pH 7.5, 90 mM KCl, 1 mM TCEP, 0.2% Tween-20), with 5mM MgCl₂ and 3

mM ATP, was added and incubated for a further 30 min at 4°C. The resin was washed with 2 mL binding buffer and eluted with 40 µL 4x NuPAGE LDS Sample Buffer (ThermoFisher). Samples were run on SDS-PAGE and stained with Coomassie blue (Expedeon).

Trypsin proteolytic digestion 0.1 mg/mL of full-length BRCA1-BARD1 was subjected to proteolytic digestion with 0.01 ng/µL trypsin (Sigma) in 25 mM Tris-HCl, pH 7.5, 90 mM KCl, 1 mM TCEP, 0.2% Tween-20. Samples were incubated at room temperature and 20 µL samples were collected at time points 0, 2, 5, 10, 15, 20, 30, 60 min and quenched with 5 µL 4x NuPAGE LDS Sample Buffer (ThermoFisher). Samples were boiled at 100°C for 5 mins and 20 µL loaded onto an SDS-PAGE gel. Gels were stained with Coomassie blue (Expedeon) and band intensities of full length BRCA1 and BARD1 was measured using ImageJ software⁵³. The rate constant of proteolysis (k_p) was determined by non-linear fitting to an exponential one phase decay curve in GraphPad Prism 8.0.2 software.

Densitometry. All densitometry was calculated using ImageJ⁵³ to quantify western blot and Coomassie-stained band intensities. For GST-PIN1-WW pull downs quantification measured the fold change of BRCA1 levels observed in the pull downs normalised to the amount of BRCA1 in the corresponding Inputs. All quantification is from at least 3-independent experiments.

For the densitometry to calculate the levels of RAD51 in the Flag- immunoprecipitation (Extended Data Fig 5e-f) we calculated the relative amounts of RAD51, BRCA1 and BARD1 in the Flag-IP from 293 FlpIn cells using densitometry performed with Image J. The amount of RAD51 enrichment was then calculated by normalising RAD51 to the combined levels of BRCA1 and BARD1 for each IP. To calculate percentage of intact protein in the trypsin proteolytic digestions, the amount of full-length protein was normalised to that at 0 min.

DR-GFP U2OS-DR3-GFP (gene conversion reporter cell line) were a generous gift from Jeremy Stark (City of Hope, Duarte USA). U2OS reporter cell lines were simultaneously co-transfected with siRNA using Dharmafect1 (Dharmacon) and DNA (RFP, or RFP-Flag-BARD1 and *I-Sce1* endonuclease expression constructs) using FuGene6 (Promega) respectively. After 16 hr the media was replaced and cells were grown for a further 48 hr before fixation in 2% PFA. RFP and GFP double positive cells were scored by FACS analysis using a CyAn flow cytometer and a minimum of 10000 cells counted. Data was analysed using Summit 6.2 software. Each individual experiment contained 3 technical repeats and normalized to siRNA controls or to WT-complemented cells. Graphs shown are combined data from 6 independent experiments and error bars show standard error. FACS gating strategy is shown in Supplementary Figure 1.

Statistics and Reproducibility. All statistics were done using two-sided unpaired *t*-test and exact *P*-values are given in each case. n.s = not-significant. All experiments were repeated to generate biological replicates. The n-value is reported for each experiment.

Supplementary Table 1 - Oligonucleotide primers used for cloning and site-directed mutagenesis

Gene	Cloning & Mutagenesis Primers	Primer Sequence
BRCA1	Y101N_F	GACACAGGTTTGGAGAATGCAAACAGC
	Y101N_R	GCTGTTTGCATTCTCCAAACCTGTGTC
	S114A_F	GGAAAATAACGCTCCTGAACATC
	S114A_R	GATGTTTCAGGAGCGTTATTTTCC
	S114D_F	GGAAAATAACGACCCTGAACATC
	S114D_R	GATGTTTCAGGGTCGTTATTTTCC
	S114P_F	GGAAAATAACCCTCCTGAACATC
	S114P_R	GATGTTTCAGGAGGGTTATTTTCC
	P115A_F (Flag-EGFP-BRCA1)	GGAAAATAACTCTGCTGAACATCTAAAAG
	P115A_R (Flag-EGFP-BRCA1)	CTTTTAGATGTTTCAGCAGAGTTATTTTCC
	P115A_F (recombinant-BRCA1)	AAACAACCTCAGCTGAGCACCT
	P115A_R (recombinant-BRCA1)	AGGTGCTCAGCTGAGTTGTTT
	P115C_F	GGAAAATAACTCTTGTAACATCTAAAAG
	P115C_R	CTTTTAGATGTTTCACAAGAGTTATTTTCC
	S114AP115A_F	GGAAAATAACGCAGCAGAACATC
	S114AP115A_R	GATGTTCTGCTGCGTTATTTTCC
	S114AP115C_F	GGAAAATAACGCTTGTAACATCTAAAAG
	S114AP115C_R	CTTTTAGATGTTTCACAAGCGTTATTTTCC
	Y179C_F	GACGTCTGTCTGCATTGAATTGGGATC
	Y179C_R	GATCCCAATTCAATGCAGACAGACGTC
	S184C_F	CATTGAATTGGGATGTGATTCTTCTGAAG
	S184C_R	CTTCAGAAGAATCACATCCCAATTCAATG
	S265Y_F	GTATCAGGGTAGTTATGTTTCAAACCTTGC
	S265Y_R	GCAAGTTTGAAACATAACTACCCTGATAC
	M1411T_F	CTCCAGCAGGAAACGGCTGAAGTAGAAGC
	M1411T_R	GCTTCTAGTTCAGCCGTTTCCTGCTGGAG
BARD1	AAE_F	CCTAGGAAAAGTTTGGCTAATGCTGAAGGAAACAAGAAGAATTC
	AAE_R	GAATTCCTCTTGTTCCTTCAGCATTAGCCAAACTTTTCCTAGG
	D135Y_F	CAGATTTGAAAGAATATAAACCTAGGAAAAG
	D135Y_R	CTTTTCCTAGGTTTATATTCTTTCAAATCTG
	K144N_F	GAAGAATTCAATTAACATGTGGTTTTTCGC
	K144N_R	GCGAAAACCCACATGTTAATTGAATTCCTC
	F147C_F	CAATTAAAATGTGGTGTTGCGCCACGTAGTAAG
	F147C_R	CTTACTACGTGGCGAACACCACATTTTAATTG
	S148A_F	CAATTAAAATGTGGTTTGCGCCACGTAGTAAG
	S148A_R	CTTACTACGTGGCGCAAACCACATTTTAATTG
PALB2	ΔNT-PALB2_F	GAAAAGATTAAGCATTCTATTAAGAAAACAG
	ΔNT-PALB2_R	CATGGTGGAGCCTGCTTT
PIN1	PIN1 WW STOP_F	CGGCCAGCGGCTAAAGCAGCAGTGG
	PIN1 WW STOP_R	CCACTGCTGCTTTAGCCGCTGGGCCG
	PIN1 W34A_F	GCCAGCCAGGCGGAGCGGCC
	PIN1 W34A_R	GGGCCGCTCCGCTGGCTGGC
	PIN1 C113S_F	GTTTCAGCGACTCCAGCTCAGCC
	PIN1 C113S_R	GGCTGAGCTGGAGTCGCTGAAC

Supplementary Table 2 - siRNA sequences

Target	siRNA Sequences	Supplier
NTC (Renilla Luciferase)	Sense: CUUACGCUGAGUACUUCGA[dT][dT] Antisense: [Phos]UCGAAGUACUCAGCGUAA G[dT][dT]	Sigma Aldrich
BARD1	Sense: UGGUUUAGCCCU CGAAGUAAG[dT][dT] Antisense: [Phos]CUUACUUCGAGGGCUAAACCA[dT][dT]	Sigma Aldrich
BRCA1 3'UTR 1	Sense: GCUCCUCUCACUCUUCAGU[dTdT] Antisense: [Phos]ACUGAAGAGUGAGAGGAGC[dT][dT]	Sigma Aldrich
BRCA1 3'UTR 2	Sense: AAGCUCCUCUCACUCUUCAGU[dT][dT] Antisense: [Phos]ACUGAAGAGUGAGAGGAGCUU[dT][dT]	Sigma Aldrich
CDK1	ON-TARGETplus SMARTpool L-003224-00-0005	Dharmacon
PALB2 3'UTR	Sense: GGAGAAUAUCUGAAUGAAUGACA [dT][dT] Antisense:[Phos]UGUCAUUCAGAUUUUCUCC [dT][dT]	Sigma Aldrich
PIN1_A	Sense: GCUCAGGCCGCCGAGUGUACUA [dT][dT] Antisense:[Phos]UAGUACACUCGGCGGCCUGAGC [dT][dT]	Sigma Aldrich
PIN1_B	Sense: GAAGACGCCUCGUUUGCGC [dT][dT] Antisense:[Phos] GCGCAAACGAGGCGUCUUC [dT][dT]	Sigma Aldrich

Supplementary Table 3 - Details of inhibitors

Inhibitor	Company	Catalogue Number	Target	Concentration
4AN	Sigma-Aldrich	A0966	PARPi	1-20 μ M
Aphidicolin	Sigma-Aldrich	A0781	DNA polymerase α	5-100 nM
Cisplatin	Sigma-Aldrich	PHR1624	DNA-crosslinker	1-5 μ M
CldU	Sigma-Aldrich	C6891	Thymidine analogue	250 μ M
Colcemid	Gibco	15212012	Microtubule poison	0.05 μ g/ml
EdU	ThermoFisher	A10044	Thymidine analogue	10 μ M
GSK Inhibitor IX	MERCK-Millipore	361552	GSK α/β	5 μ M
Hydroxyurea	Sigma-Aldrich	H8627	Ribonucleoside reductase	1-10 mM
IdU	Sigma-Aldrich	I7125	Thymidine analogue	25 μ M
INDY	MERCK-Millipore	405273	DYRK	20 μ M
Juglone	Sigma-Aldrich	H47003	PIN1	20 μ M
MG132	Sigma-Aldrich	474787	Proteasome inhibitor	5 μ M
Mirin	Sigma-Aldrich	M9948	MRE11	50 μ M
Olaparib	Selleckchem	S1060	PARPi	1-20 μ M
RO-3306	Selleckchem	S7747	CDK1/2	5 μ M
Roscovitine	Sigma-Aldrich	R7772	CDK1/2	25 μ M
SP600125	Selleckchem	S1460	JNK1/2	25 μ M
U0126	Selleckchem	S1102	MEK1/2	10 μ M
Veliparib	Selleckchem	S1004	PARPi	1-20 μ M

Supplementary Table 4 - Antibody Table

Antibody (clone)	Host	Supplier	Cat. number	Lot number	Technique	Conc	RRID
β-actin	Rabbit	Abcam	Ab8227	GR3215935-1	WB	1:3000	AB_2305186
BARD1	Rabbit	Abcam	ab226854	GR3197067-8	WB	1:2000	
BRCA1 (D-9)	Mouse	Santa Cruz	Sc6954	C2519	IF	1:500	AB_626761
BRCA1 (MS110)	Mouse	MERCK Millipore	OP94	3091924	WB	1:500	AB_213438
pS114-BRCA1 (3C10G8)	Mouse	Genscript	Custom design		WB	1:1000	
pS114-BRCA1	Rabbit	Genscript	Custom design		WB/PLA	1:1000	
CDK1 (A17)	Mouse	Abcam	Ab18	GR133813-4	WB	1:2000	AB_2074906
CldU (BrdU)	Rat	Abcam	Ab6326	GR3173537-9	Fibres/IF	1:2000	AB_305426
Flag (M2)	Mouse	SigmaAldrich	F1804	SLBT7654	WB	1:1000	AB_262044
IdU (BrdU)	Mouse	BD Biosciences	347580	8151735	Fibres	1:500	AB_400326
γH2AX	Rabbit	Abcam	Ab2893	GR3242597-1	IF	1:2000	AB_303388
PALB2	Rabbit	Bethyl	A301.246A		WB	1:2000	AB_890607
PIN1	Mouse	R&D Systems	MAB2294		WB	1:1000	AB_2163944
RAD51 (H-92)	Rabbit	Santa Cruz	SC8349	E0616	WB/IF	1:1000	AB_2253533
RAD51 (Ab-1)	Rabbit	Calbiochem	PC130	3135376	PLA	1:100	AB_2238184
Tubulin	Mouse	Santa Cruz	sc-5286	H0613	WB	1:5000	AB_628411
Vinculin [EPR8185]	Rabbit	Abcam	Ab129002	GR221671-50	WB	1:2000	AB_11144129
BrdU-Biotin	Mouse	Jackson ImmunoResearch	200-002-211	134961	PLA	1:100	AB_2339006
BrdU-Biotin	Rabbit	Bethyl	A150-109A		PLA	1:100	AB_67327
Donkey α Mouse AlexaFluor 488	Donkey	Life technologies	A21202	1975519	IF	1:5000	AB_141607
Donkey α Rabbit AlexaFluor 488	Donkey	Life technologies	A21206	1874771	IF	1:5000	AB_2535792
Donkey α Mouse AlexaFluor 555	Donkey	Life technologies	A31570	1774719	IF	1:5000	AB_2536180
Donkey α Rabbit AlexaFluor 555	Donkey	Life technologies	A31572	1945911	IF	1:5000	AB_162543
Donkey α rat AlexaFluor 555	Donkey	Life technologies	A21434	1987272	IF	1:5000	AB_2535855
Rabbit α Mouse HRP	Rabbit	Dako	P0161	20062080	WB	1:10000	AB_2687969
Swine α Rabbit HRP	Swine	Dako	P0217	20047666	WB	1:10000	AB_2728719

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author contributions: M. D-M generated reagents, performed survival, PLA, fibre experiments and *in vitro* analysis. R.M.D generated reagents, performed fibre experiments, *in vitro* RAD51 assays, and survival analysis. K.S performed metaphases and long-term P115A analysis. M.J generated purified proteins and performed *in vitro* analysis. T.M. generated full length BRCA1-BARD1 and performed initial analysis. S.T. purified full length BRCA1-BARD1 and performed *in vitro* RAD51 binding and trypsin digestion. G.E.R performed western blots and Flag-IPs. A.C performed GST-PIN1-WW analyses. H.L.M. performed pS114-EdU PLA assay. A.K.W. analysed RAD51 foci (BRCA1 variants). H.R.S analysed RAD51 foci (BARD1 variants). J.B provided technical support. J.C made M1411T cells and Cisplatin survival analysis. A.J.G. generated constructs and cell lines. G.S. made preliminary observations. X.Z supervised S.T. and T.M. J.R.M, R.M.D and M. D-M wrote the paper. All authors commented on the paper and research. R.M.D and J.R.M conceived and directed the project.

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The authors declare no competing interests.

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Data Availability The datasets generated during the current study are available from the corresponding authors on reasonable request.

Extended Data Figure Legends

Extended Data Figure 1. The canonical BRCA1-PALB2 interaction is not required for fork protection

a, Schematic of the DNA fibre assay used to measure fork protection by calculating IdU/CldU ratios

b-c, IdU/CldU ratios from U2OS cells depleted for BRCA1 or PALB2 treated with HU (5 mM, 3 hours). n=300 fibres from 3 biological repeats, bars=median±95% CI. Representative western blot (n=3, **c**).

d, Schematic of BRCA1 protein indicating RING (red), RAD51-binding (green), coiled-coil (blue) and BRCT repeat (purple) domains. The M1411T patient variant disrupts PALB2 binding and is located in the coiled-coil domain.

e, Schematic of PALB2 protein indicating BRCA1-interacting coiled-coil (blue), ChAM and DNA binding (purple and green) and WD40-like repeat (orange) domains. The ΔNT-PALB2 mutant lacks the N-terminal coiled-coil domain.

f, Colony survival following cisplatin treatment (2.5 μM, 2 hours) in HeLa cells depleted for BRCA1 and complemented with Flag-EGFP-WT-BRCA1 or Flag-EGFP-M1411T-BRCA1. Data=mean±SEM, n=4.

g, Colony survival following cisplatin treatment (2 hours) in U2OS cells depleted for PALB2 and complemented with Flag-WT-PALB2 or Flag-ΔNT-PALB2. Data=mean±SEM, n=4.

h, Representative western blot for **j** (n=3).

i, Representative Western blot for **k** (n=3)

j, IdU/CldU ratios from U2OS cells depleted for BRCA1 and complemented with WT or Flag-EGFP-M1411T-BRCA1 and treated with HU (5 mM, 3 hours). n=300 fibres from 3 biological repeats, bars=median±95% CI.

k, IdU/CldU ratios from U2OS cells depleted for PALB2 and complemented with WT or Flag- ΔNT-PALB2 and treated HU (5 mM, 3 hours). n=300 fibres from 3 biological repeats, bars=median±95% CI.

l, The % stalled replication forks in U2OS cells depleted for BRCA1 and complemented with Flag-EGFP-WT-BRCA1 or Flag-EGFP-M1411T-BRCA1. Data=mean±SEM, n=3.

m, As for **l** but depleted for PALB2 and complemented with Flag-WT-PALB2 (n=3) or Flag-ΔNT-PALB2 (n=3). Data=mean±SEM, n=2 (NTC, siPALB2).

n, The % replication forks able to restart after release from HU (5 mM, 3 hours) in U2OS cells depleted for BRCA1 and complemented with Flag-EGFP-WT-BRCA1 or Flag-EGFP-M1411T-BRCA1. Data=mean±SEM, n=3.

o, As for **n** but depleted for PALB2 and complemented with Flag-WT-PALB2 (n=3) or Flag-ΔNT-PALB2 (n=3). Data=mean±SEM, n=2 (NTC, siPALB2).

All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 2. BRCA1-BARD1 mediated fork protection requires the BARD1-RAD51 binding domain

a-b, Colony survival following Olaparib treatment (2 hours, n=4) (**a**) or HU (16 hours, n=7) (**b**) in U2OS cells depleted for BARD1 and complemented with RFP-Flag-WT-BARD1 or RFP-Flag-AAE-BARD1. Data=mean±SEM.

c-d, IdU/CldU ratios from U2OS cells depleted for BARD1 and complemented with WT or RFP-Flag-AAE-BARD1 and treated with HU (5 mM HU, 3 hours). n=315 fibres from 3 biological repeats, bars=median±95% CI. Representative western blot (n=3, **d**).

e-f, As for **c** but complemented with WT or RFP-Flag-R99E-BARD1. n=300 fibres from 3 biological repeats, bars=median±95% CI. Representative western blot (n=3, **f**).

All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 3. The role of RING-proximal BRCA1-BARD1 phospho-sites in replication stress

a-e, IdU/CldU ratios (**a**) and CldU track lengths (**b,d**) from U2OS cells depleted for BARD1, complemented with RFP-Flag-BARD1 variants and treated with HU (5 mM, 3 hours). n=300 (**a**), n=600 (**b**) fibres from 3 biological repeats or n=290 from 2 biological repeats (**d**). Bars=median±95% CI. Representative western blots (n=3, **c**) (n=2, **e**).

f, The percentage of co-localising BRCA1-CldU foci per cell was scored from U2OS cells expressing Flag-EGFP-WT-BRCA1 or Flag-EGFP-S114A-BRCA1. n=25 cells, bars=median±95% CI. ns=not significant.

g, Representative blot of Flag immunoprecipitation of Flag-EGFP-BRCA1 variants from HEK293 cells (n=3).

h-i, As for **b**, but depleted for BRCA1 and complemented with Flag-EGFP-S114A-BRCA1 and treated with Mirin (50 µM) and HU (5 mM) for 3 hours. n=245 fibres from 3 biological repeats, bars=median±95% CI. Representative western blot (n=3, **i**).

All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 4. Phosphorylation of BRCA1 at serine 114 promotes PIN1 interaction

a, Schematic of the protein structure of PIN1 indicating phospho-binding WW and Peptidyl-Prolyl-Isomerase (PPIase) domains. Cartoons illustrate the GST-fusions of the WW domain WT and W34A phospho-binding mutant. Coomassie blots indicate recombinant GST-WW fragments purified from *E. coli*.

b, Densitometry quantification of GST-WW pull downs from U2OS cells expressing Flag-EGFP-WT-BRCA1 and Flag-EGFP-S114A-BRCA1. Beads bound by GST-W34A WW-domain were used as a negative control from 4-independent experiments. Data=mean±SEM. All *P*-values derived from two-sided unpaired *t*-test. Representative image shown in Fig 1e.

c, GST-WW pull downs from U2OS cells expressing Flag-EGFP-WT-BRCA1 and Flag-EGFP-S114D-BRCA1. Beads bound by GST-W34A WW-domain were used as a negative control. Representative blot (n=3).

d, As for **c** but with cells expressing Flag-EGFP-WT-BRCA1 and treated ± HU (5 mM, 3 hours). Representative blot (n=3).

e, As for **d** but in HEK293 cells and probed for endogenous BRCA1. The final lane indicates lysates pre-treated with calf-intestinal phosphatase. Representative blot (n=3).

f, As for **c** but from U2OS cells expressing RFP-Flag-WT-BARD and RFP-Flag-S148A-BARD1. Representative blot (n=3).

g, Table of inhibitors used to inhibit proline-directed kinases in **h**.

h, GST-WW pull downs from HEK293 cells expressing Flag-EGFP-BRCA1 treated with and without kinase inhibitors described in **g**. Beads bound by GST-W34A WW-domain were used as a negative control. Representative blot (n=3).

i, GST-WW pull downs from HEK293 cells expressing Flag-EGFP-BRCA1 and depleted for CDK1.

j, Coomassie blots of purified recombinant WT and S114A His-BRCA1₁₋₃₀₀:His-BARD1₂₆₋₁₄₂ (representative gel n=3).

k, Recombinant purified His-BRCA1₁₋₃₀₀:His-BARD1₂₆₋₁₄₂ were incubated with recombinant active CDK1/Cyclin A2, CDK2/Cyclin A or CDK9/Cyclin K. Western blots were probed for phospho-S114-BRCA1 (3C10G8) and BRCA1 (representative blot n=3).

I, Recombinant purified WT or S114A His-BRCA1₁₋₃₀₀:His-BARD1₂₆₋₁₄₂ were incubated with recombinant active CDK2/Cyclin A. Western blots were probed for phospho-S114-BRCA1 (3C10G8) and BRCA1 (representative blot n=3).

Extended Data Figure 5. PIN1 regulates the BRCA1-BARD1 heterodimer to promote fork protection

a, CldU fibre tract lengths were measured from U2OS cells depleted for BRCA1 and treated with HU (5 mM) and Juglone (20 μ M) for 3 hours. n=450 fibres from 3 biological repeats, bars=median \pm 95% CI.

b-c, CldU fibre tract lengths were measured from U2OS in cells depleted for BARD1 and/or PIN1 and treated with HU (5 mM, 3 hours). n=300 fibres from 2 biological repeats, bars=median \pm 95% CI. Representative western blot (n=2, **c**).

d-e, Representative blot (**d**) and quantification (**e**) of BRCA1 expression levels normalised to β -actin levels following PIN1 depletion from 9-biological repeats. Data=mean \pm SEM, n=9. n.s = not significant.

f-g, IdU/CldU ratios from U2OS cells depleted for BRCA1 and complemented with Flag-EGFP-BRCA1 variants and treated with HU (5 mM, 3 hours). n=200 fibres from 2 biological repeats, bars=median \pm 95% CI. Representative western blot (n=2, **g**).

All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 6. BRCA1-BARD1 isomerisation enhances RAD51 binding

a, WT and S114D His-BRCA1₁₋₅₀₀ and BARD1₂₇₋₃₂₇ incubated with full length GST-PIN1 to induce isomerisation. His-BRCA1-BARD1 complexes were then incubated with recombinant active RAD51 and their ability to bind RAD51 was assessed by His-purification of the complex followed by western blot. Representative image (n=2).

b, Representative Coomassie gel of recombinant purified full length WT and C113S GST-PIN1 from *E. coli* (n=2).

c, Representative Coomassie gel of recombinant purified His-BRCA1₁₋₅₀₀ and BARD1₂₇₋₃₂₇ from *E. coli*. (n=3).

d, Recombinant RAD51 incubated with recombinant full length BRCA1-BARD1 mutants in the presence of ATP and Mg²⁺. The ability of BRCA1-BARD1 to bind RAD51 was assessed by Strep-pull down of BRCA1 followed by SDS-PAGE and Coomassie staining (n=2).

e, Quantification of recombinant RAD51 bound to recombinant full length BRCA1-BARD1 and P115A-BRCA1-BARD1 mutant in the presence of ATP and Mg²⁺, normalised to the respective amount of BRCA1 and BARD1 and given relative to WT BRCA1-BARD1 to indicate fold change. Data=mean \pm SEM. n=6 (2 biological repeats each with 3 technical repeats).

f-g, Quantification of amount of RAD51 co-immunoprecipitated with Flag-EGFP-BRCA1 and RFP-Flag-BARD1 from HEK293 cells, normalised to BRCA1-BARD1 levels precipitated. Data=mean \pm SEM, n=12. Representative image (**g**).

h, Representative gel for recombinant full length BRCA1-BARD1 incubated with trypsin and samples taken at the times indicated. The limited proteolysis profiles were assessed by SDS-PAGE and Coomassie staining. n=3.

i, Representative images for Fig 3e. RAD51 co-localisation with nascent DNA, marked by pulse labelling with EdU, was measured using the proximity ligation assay (PLA) in U2OS cells depleted for BRCA1 and complemented with Flag-EGFP-BRCA1 variants as indicated. Red foci indicate RAD51/EdU-Biotin interaction in cells. Scale bars=10 μ m.

All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 7. BRCA1-S114A shows increased sensitivity to replication stress agents

a-d, Colony survival following treatment with Aphidicolin (16 hours) (**a**) or PARP inhibitors (2 hours) Olaparib (**b**), Veliparib (**c**) and 4AN (**d**) was measured in HeLa cells depleted for BRCA1 and complemented with Flag-EGFP-WT-BRCA1 or Flag-EGFP-S114A-BRCA1. Data=mean± SEM, n-values given in brackets for each condition.

e, Colony survival following 16-hour treatment with HU was measured in U2OS cells depleted for BRCA1 and complemented with WT or S114A Flag-EGFP-BRCA1. Data=mean± SEM, n=3.

f-g, RAD51 foci in S-phase U2OS cells marked by EdU were scored from BRCA1 depleted cells complemented with Flag-EGFP-WT-BRCA1 or Flag-EGFP-S114A-BRCA1 treated with 20 μM Olaparib (2 hours). Representative images with scale bars = 10 μm (**f**). Graph shows number of RAD51 foci/EdU-positive cell (**g**). Bars=median±95% CI, n-values given in brackets for each condition. *P*-value derived from two-sided unpaired *t*-test.

Extended Data Figure 8. Constitutive expression of Flag-EGFP-P115A-BRCA1 promotes DNA damage

a, U2OS cells induced to constitutively express Flag-EGFP-WT-BRCA1 or Flag-EGFP-P115A-BRCA1 were stained for γH2AX foci as a marker for DNA damage accumulation over time. γH2AX foci were scored from EdU negative cells. Combined data from 3-biological repeats. n=90 cells. Bars=mean±SEM. *P*-value derived from two-sided unpaired *t*-test.

Extended Data Figure 9. Patient variants define a novel region of BRCA1 required for fork protection

a, Colony survival following 2 hour treatment with Olaparib was measured in cells depleted for BRCA1 and complemented with WT or Flag-EGFP-BRCA1 variants (Y101N, Y179C, S184C, S265Y in U2OS; S114P and R133C in HeLa). Data=mean±SEM. n-values given in brackets for each condition

b-c, As for **a**, but with continuous treatment of Olaparib (**b**) or cisplatin (**c**) and all patient variants are in U2OS cells.

d-e, RAD51 foci formation was scored at 2 hours following 2 Gy IR in U2OS cells depleted for BRCA1 and complemented with WT or patient variant Flag-EGFP-BRCA1 as indicated. Representative images for **e**. Scale bars=10μm (**d**). Graph scored n=150 cells from 3 biological repeats. Bars=median±95% CI (**e**). All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 10. Patient variants define a novel region of BARD1 required for fork protection

a, Colony survival following 2 hour treatment with Olaparib was measured in U2OS cells depleted for BARD1 and complemented with WT or patient variant RFP-Flag-BARD1 as indicated.

Data=mean±SEM. n-values given in brackets for each condition

b-c, As for **a** but with continuous treatment with Olaparib (**b**) or cisplatin (**c**).

d-e, RAD51 foci formation was scored at 2 hours following 2 Gy IR in U2OS cells depleted for BARD1 and complemented with WT or patient variant RFP-Flag-BARD1 as indicated. Representative images (**d**). Scale bars=10μm. Graphed data is combined from 3-biological repeats (**e**). Actual n-values for each condition given in brackets. Bars=median±95% CI.

f, HR (U2OS DR3-GFP) assays using siBARD1 or siNTC treated cells transfected with *I-SceI* and RFP, or RFP-Flag-BARD1 constructs shown and counted by FACS analysis. GFP+ cells were normalised to RFP-positive cells as a measure of transfection efficiency. %-repair is given relative to siNTC. Data=mean±SEM, n=6. Gating strategy is describe in Supplemental Figure 1. All *P*-values derived from two-sided unpaired *t*-test.

Extended Data Figure 11. Isomerisation of phospho-BRCA1-BARD1 promotes replication fork protection

Model to illustrate CDK1/2 (grey) phosphorylation at S114 (red) and subsequent PIN1 (purple) isomerisation events on BRCA1 (green) and BARD1 (orange). BRCA1 isomerisation enhances the ability of BARD1 to associate with RAD51 (brown) to promote replication fork protection

Extended Data Table 1. DNA damage survival and fork protection responses by BRCA1-BARD1 variants

A summary of the main findings from this study.

Further References

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